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Martine PLANCHE

NATIONAL INSTITUTE OF INDUSTRIAL PROPERTY
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REGISTERED OFFICE
26 bis, rue de Saint Petersbourg
75800 PARIS Cédex 08
Telephone: 01 53 04 53 04
Telefax: 01 42 93 59 30

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NIIP

NATIONAL INSTITUTE
OF INDUSTRIAL PROPERTY

26 bis, rue de Saint Pétersbourg
75800 Paris Cedex 08
Telephone: (1) 42.94.52.52
Telefax: (1) 42.93.59.30

PATENT, UTILITY CERTIFICATE

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NATIONAL REGISTRATION No. 97/05,608

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1. NAME AND ADDRESS OF THE APPLICANT OR THE REPRESENTATIVE TO WHOM THE CORRESPONDENCE IS TO BE ADDRESSED

Marie-Pauline AYROLES
Intellectual Property Directorate
PASTEUR MARIEUX Sérums et Vaccins
58, avenue Leclerc
69007 LYON

No. of permanent power of attorney	Correspondent's references	Telephone
PG 04852	PM 97/006	04 72 73 79 31

2 APPLICATION Nature of the industrial property right

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Title of the invention (maximum 200 characters)

Anti-Helicobacter vaccine composition comprising a Th1-type adjuvant.

3 APPLICANT(S) SIREN No. 3.4.9.5.0.5.3.7.0 APE-NAF code 2.4.4.C

Name and forenames (underline the surname) or company name

PASTEUR MERIEUX Sérums et Vaccins S.A.

Legal form

S.A.

Nationality/Nationalities French

Full address(es)

58, avenue Leclerc
69007 LYON

Country

FRANCE

If insufficient space, continue on plain paper

4 INVENTOR(S) The inventors are the applicants [ ] yes [X] no If the answer is no, provide a separate designation

5 REDUCTION OF THE RATE OF FEES [ ] requested for the first time [ ] requested prior to filing; attach copy of the favourable decision

6 PRIORITY DECLARATION OR APPLICATION FOR THE BENEFIT OF THE FILING DATE OF A PRIOR APPLICATION

Country of origin	Number	Filing date	Nature of the application

7 DIVISIONS previous to the present application No. date No. date

8 SIGNATURE OF THE APPLICANT OR REPRESENTATIVE (name and capacity of the signatory - registration No.)

(signature)
Marie-Pauline AYROLES
Intellectual Property Manager

SIGNATURE OF THE RECEIVING OFFICIAL

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DESIGNATION OF THE INVENTOR
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PATENTS ADMINISTRATIVE DIVISION

26bis, rue de Saint-Pétersbourg
75800 Paris Cédex 08
Tel: (1) 42 94 52 52 - Telefax: (1) 42 93 59 30

NATIONAL REGISTRATION No.

97/05,608

TITLE OF THE INVENTION:

Anti-Helicobacter vaccine composition comprising a Th1-type adjuvant.

THE UNDERSIGNED

PASTEUR MERIEUX Sérums et Vaccins

DESIGNATE(S) AS INVENTOR(S) (State name, forenames and address and underline surname):

Bruno GUY
15B, rue des Noyers
69005 LYON (FR)

Jean HAENSLER
17, rue Piccandet
69290 Saint GENIS les OLLIERES (FR)

NOTE: In exceptional cases, the name of the inventor may be followed by that of the company to which he belongs (membership company) when the latter is other than the company which is the applicant or titleholder.

Date and signature(s) of the applicant(s) or of the representative

30 April 1997
(signature)
Marie-Pauline AYROLES

BA 113/270195

The subject of the present invention is the specific use of a vaccine preparation intended to induce, in a mammal, a protective immune response against a pathogenic organism infecting the mucous membranes, in particular against *Helicobacter* bacteria.

Helicobacter is a bacterial genus characterized by Gram-negative helical bacteria. Several species colonize the gastrointestinal tract of mammals. There may be mentioned in particular *H. pylori*, *H. heilmanii*, *H. felis* and *H. mustelae*. Although *H. pylori* is the species most commonly associated with human infections, in some rare cases, it has been possible to isolate in man *H. heilmanii* and *H. felis*. A bacterium of the *Helicobacter* type, *Gastrospirillum hominis*, has also been described in man.

Helicobacter infects more than 50% of the adult population in developed countries and nearly 100% of that of developing countries, thereby making it one of the predominant infectious agents worldwide.

H. pylori is so far exclusively found at the surface of the mucous membrane of the stomach in man and more particularly around the crater lesions of gastric and duodenal ulcers. This bacterium is currently recognized as the aetiological agent of antral gastritis and appears as one of the cofactors required for the development of ulcers. Moreover, it seems that the development of gastric carcinomas may be associated with the presence of *H. pylori*.

It therefore appears to be highly desirable to develop a vaccine intended to prevent or treat *Helicobacter* infections.

To date, several *Helicobacter* proteins have already been proposed as vaccinal antigen and the method of vaccination which is commonly recommended consists in delivering the antigen at the level of the gastric mucous membrane, that is to say at the very site where the immune response is desired. To do this, oral administration was therefore selected.

Still with the same aim (induction of an immune response at the level of the stomach), it has been more recently proposed to deliver the antigen at a mucous site other than the gastric mucous membrane, such as the nasal or rectal mucous membrane for example (WO 96/31235). Lymphocytes stimulated by the antigen in a so-called inducer mucosal territory can migrate and circulate selectively so as to go and induce an immune response in other so-called effecter mucosal territories.

A variant of these methods consists in carrying out a first immunization by the systemic route before administering the antigen by the nasal route.

For administration by the mucosal route, the antigen, most often a bacterial lysate or a purified protein, is combined with an appropriate adjuvant such as the cholera toxin (CT) or the heat-labile toxin (LT) from *E. coli*.

When the administration by the mucosal route is used, the humoral response which is observed is predominantly of the IgA type. This indeed indicates that there has been a local immune response.

Some authors thought very early on that there was a good correlation between a strong response of the IgA type and a protective effect (Czinn et al., Vaccine (1993) 11: 637). Others gave a more reserved opinion (Bogstedt et al., Clin. Exp. Immunol. (1996) 105: 202). Although there is up until now no real certainty as regards this subject, the induction of antibodies which are in particular of the IgA type appears nonetheless desirable for most authors.

In general, the appearance of IgAs is indicative of the coming into play of a response on the part of the type 2 T helper lymphocytes (Th2 response).

Indeed, the stimulation of the T helper lymphocytes by a particular antigen makes it possible to obtain various subpopulations of T helper cells, characterized by different cytokine synthesis profiles.

The Th1 cells in particular produce selectively interleukin-2 (IL-2) and interferon-γ (IFN-γ), whereas the Th2 cells secrete preferably IL-4, -5 and -10. Because of their differentiated production of cytokines, these two types of T helper cells have distinct roles: the Th1 cells promote cell-mediated immunity *i.a.* an inflammatory-type response, whereas the Th2 cells stimulate humoral response of the IgA, IgE and certain IgG subclass types. It is also known that the cytokines produced by mouse Th1 cells can stimulate antibody response and in particular that IFN-γ induces an IgG2a response.

Thus, from the various studies in the prior art, the view emerges according to which the induction of a Th2 response characterized by the appearance of IgA is essential, if not enough, to obtain a protective effect.

Surprisingly, it has now been discovered that even if a Th2 response is not damaging, it is also necessary to induce a high Th1 response. Indeed, experimental results now demonstrate that a protective effect may be more easily correlated with a Th1 response than with a Th2 response.

Contrary to what was initially sought (D'Elios et al., J. Immunol. (1997) 158: 962), the present application therefore reveals the importance of inducing an inflammatory-type Th1 response at the time of immunization, without which a protective effect cannot be observed.

It is possible to induce a Th1 response against *Helicobacter* by adjusting a number of factors, such as, for example, the type of adjuvant. It has indeed been demonstrated that by using certain adjuvants, a level of protection can be obtained which is similar to or greater than that observed when the mucosal route and adjuvants such as bacterial toxins are used.

Consequently, the subject of the present invention is:

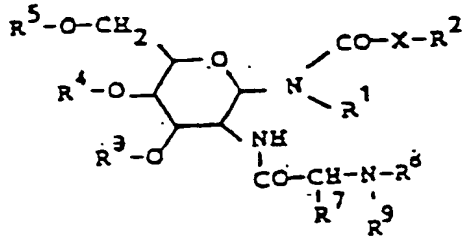
(a) The conjoint use of an immunogenic agent derived from *Helicobacter* and of a compound capable of promoting the induction of a T helper 1 (Th1) type immune response against *Helicobacter*, in the manufacture of a medicament intended to be administered by the systemic route to prevent or treat a *Helicobacter* infection.

(b) A pharmaceutical composition which comprises an immunogenic agent derived from *Helicobacter* and at least one compound (capable of promoting the induction of a T helper 1 (Th1) type immune response against *Helicobacter*) selected from:

(i) saponins purified from an extract of *Quillaja saponaria*;

(ii) cationic lipids which are weak inhibitors of protein kinase C and have a structure which includes a lipophilic group derived from cholesterol, a bonding group selected from carboxyamides and carbamoyls, a spacer arm consisting of a branched or unbranched linear alkyl chain of 1 to 20 carbon atoms, and a cationic amine group selected from primary, secondary, tertiary and quaternary amines; and

(iii) glycolipopeptides of formula (I):



(I)

in which:

R^1 represents an alkyl residue saturated or unsaturated once or several times and comprising from 1 to 50 carbon atoms, preferably 1 to 20 carbon atoms,

X represents $-CH_2-$, -O- or -NH-,

R^2 represents a hydrogen atom or an alkyl residue saturated or unsaturated once or several times and comprising from 1 to 50 carbon atoms, preferably 1 to 20 carbon atoms,

R^3, R^4 and R^5 each represent, independently of each other, a hydrogen atom or an acyl-CO-R^6 residue in which R^6 represents an alkyl residue having from 1 to 10 carbon atoms,

R^7 represents a hydrogen atom, a C_1-C_7 alkyl, hydroxymethyl, 1-hydroxyethyl, mercaptomethyl, 2-(methylthio)ethyl, 3-aminopropyl, 3-ureidopropyl, 3-guanidylpropyl, 4-aminobutyl, carboxymethyl, carbamoylmethyl, 2-carboxyethyl, 2-carbamoylethyl, benzyl, 4-hydroxybenzyl, 3-indolylmethyl or 4-imidazolylmethyl group,

R^8 represents a hydrogen atom or a methyl group, and

R^9 represents a hydrogen atom, an acetyl, benzoyl, trichloroacetyl, trifluoroacetyl, methoxycarbonyl, t-butyloxycarbonyl or benzyloxycarbonyl group, and

R^7 and R^8 may, when they are taken together, represent a $-CH_2-CH_2-CH_2-$ group.

(c) The use of an immunogenic agent derived from *Helicobacter* and of at least one compound selected

from the compounds (i) to (iii) cited above, in the manufacture of a pharmaceutical composition capable of inducing a T helper 1 (Th1) type immune response against *Helicobacter*; and

(d) A method for preventing or treating an infection promoted by a microorganism capable of infecting the gastroduodenal mucous membrane of a mammal, *e.g.* a *Helicobacter* infection, according to which there is administered to the mammal by the systemic route, in one or more applications, a composition containing at least one immunogenic agent derived from the said microorganism, *e.g.* from *Helicobacter*, and at least one compound capable of promoting the induction of a T helper 1 (Th1) type immune response against *e.g.* *Helicobacter*.

(e) A method for preventing or treating an infection promoted by a microorganism capable of infecting the gastroduodenal mucous membrane of a mammal, *e.g.* a *Helicobacter* infection, according to which there is administered to the mammal, in one or more applications, a composition containing at least one immunogenic agent derived from the said microorganism, *e.g.* from *Helicobacter*, and at least one compound selected from the compounds (i) to (iii) cited above, and by which a Th1-type immune response is induced against *e.g.* *Helicobacter*.

The induction of a useful Th1 response can be demonstrated for the purposes of the present invention by estimating the relative level of the Th1 response relative to the Th2 response, by comparing, for example, the IgG2a and IgG1 levels induced in mice against *Helicobacter*, which are respectively indicative of the coming into play of the Th1 and Th2 responses. Indeed, the Th1 response which is sought is generally accompanied by a Th2 response. However, it is

considered that the Th2 response should not be significantly predominant relative to the Th1 response. The IgG2a and IgG1 levels induced in mice can be assessed conventionally using an ELISA test, provided that the tests used for each of the two subisotypes are of the same sensitivity and, in particular, that the anti-IgG2a and anti-IgG1 antibodies are of the same affinity.

The quantities of IgG2a and IgG1 may be measured in particular using an ELISA test which is identical or similar to that described below. The wells of a polycarbonate ELISA plate are coated with 100 µl of a bacterial extract from *Helicobacter, e.g. H. pylori*, at about 10 µg/ml in carbonate buffer. The ELISA plate is incubated for 2 hours at 37°C and then overnight at 4°C. The plate is washed with PBS buffer (phosphate buffer saline) containing 0.05% Tween 20 (PBS/Tween buffer). The wells are saturated with 250 µl of PBS containing 1% bovine serum albumin in order to prevent nonspecific binding of the antibodies. After incubating for one hour at 37°C, the plate is washed with PBS/Tween buffer. The antiserum collected from mice, a number of days after the latter have received the composition intended to induce a Th1-type immune response against *Helicobacter*, is serially diluted in PBS/Tween buffer. 100 µl of the dilutions are added to the wells. The plate is incubated for 90 minutes at 37°C, washed and evaluated according to standard procedures. For example, a goat antibody to mouse IgG2a or IgG1, coupled to an enzyme such as peroxidase, is used. The incubation in the presence of this antibody is continued for 90 minutes at 37°C. The plate is washed and then the reaction is developed with the appropriate substrate (for example O-phenyldiamine dihydrochloride when the enzyme used is peroxidase). The reaction is evaluated by colorimetry (by measuring the absorbance by spectrophotometry). The IgG2a or IgG1 titre of the antiserum corresponds to the reciprocal of the dilution giving an absorbance of 1.5 at 490 nm.

The induction of a useful Th1 response for the purposes of the present invention is marked by a ratio of the ELISA IgG2a : IgG1 titres in mice which should be greater than 1/100, 1/50 or 1/20, advantageously greater than 1/10, preferably greater than 1/3, most preferably greater than 1/2, 5 or 10. When this ratio is around 1, the Th1/Th2 response is said to be mixed or balanced. When the ratio is greater than or equal to 5, the Th1 response is then said to be preponderant.

The production of a Th1 (or Th2) response in mice is predictive of a Th1 (or Th2) response in man. Although it is easier to evaluate the type of response in mice, it can also be done in man by measuring the levels of cytokines specific for the Th1 response on the one hand and, on the other hand, for the Th2 response, which are subsequently induced. The Th1 and Th2 responses can be evaluated directly in man relative to each other on the basis of the levels of cytokines specific for the two types of response (see above) *e.g.* on the basis of the IFN-γ/IL-4 ratio.

Alternatively, if the assay method described above is used, it is possible to predict that the ELISA titre which reflects the quantity of IgG2a should be equal to or greater than 10,000, preferably equal to or greater than 100,000, in a particularly preferred manner equal to or greater than 1,000,000; this then means that the Th1 response is significant.

The mammal for which the pharmaceutical composition or the method is intended is advantageously a primate, preferably a human.

According to an advantageous mode, at least two compounds are used, one being selected from the saponins purified from an extract of *Quillaja saponaria* and the other being selected from cationic lipids which are weak inhibitors of protein kinase C and have a structure which includes a lipophilic group derived from cholesterol, a bonding group selected from carboxyamides and carbamoyls, a spacer arm consisting of a branched or unbranched linear alkyl chain of 1 to

20 carbon atoms, and a cationic amine group selected from primary, secondary, tertiary and quaternary amines.

Saponins useful for the purposes of the present invention are described in particular in US Patent No. 5,057,540 with reference not to their structures but to the fractions in which they are present after fractionation of an aqueous extract of *Quillaja saponaria Molina* bark by high-performance liquid chromatography (HPLC) and low-pressure chromatography on silica. In particular, the fractions QA-7, QA-17, QA-18 and QA-21 also called QS-21 may be mentioned. The use of the latter is particularly advantageous. QS-21 is known to be an adjuvant which promotes the induction of a predominantly Th1-type immune response. The adjuvant is then said to be of the Th1 type.

Useful cationic lipids for the purposes of the present invention are in particular described in US Patent No. 5,283,185. By way of example, there may be mentioned cholesteryl-3β-carboxylamidoethylenetrimethylammonium iodide, 1-dimethylamino-3-trimethylammonio-DL-2-propylcholesterylcarboxylate iodide, cholesteryl-3β-carboxyamidoethyleneamine iodide, cholesteryl-3β-oxysuccinamidoethylenetrimethylammonium iodide, 1-dimethylamino-3-trimethylammonio-DL-2-propylcholesteryl-3β-oxysuccinate iodide, 2-[(2-trimethylammonio)ethylmethylamino]ethylcholesteryl-3β-oxysuccinate iodide, 3β-[N-(polyethyleneimine)carbamoyl]-cholesterol and 3β-[N-(N',N'-dimethylaminoethane)-carbamoyl]cholesterol (DC-chol); the latter being particularly advantageous. DC-chol is known to be an adjuvant which promotes the induction of a Th1/Th2 type mixed balanced response. The adjuvant is then said to be of the Th1/Th2 or Th1 + Th2 type.

These cationic lipids may be used in dispersion or alternatively made in the form of liposomes. Liposomes may be made as described in US Patent No. 5,283,185, by combining cationic lipids with a neutral

phospholipid, *e.g.* phosphatidylcholine or phosphatidylethanolamine.

Useful glycolipopeptides for the purposes of the present invention are in particular described in US Patent No. 4,855,283 and EP 206,037. They are in particular glycolipids of general formula (I) in which a sugar residue is a 2-amino-2-deoxy-D-glucose or 2-amino-2-deoxy-D-galactose residue. The 2-amino group of the amino sugar may be linked to glycine, sarcosine, hippuric acid, alanine, valine, leucine, isoleucine, serine, threonine, cysteine, methionine, ornithine, citrulline, arginine, aspartic acid, asparagine, glutamic acid, glutamine, phenylalanine, tyrosine, proline, tryptophan or histidine in the D or L form with aminocarboxylic acids such as α-aminobutyric acid, α-aminovalerianic acid, α-aminocaproic acid or α-aminoheptanoic acid in the D form or in the L form.

More particularly, the following glycolipopeptides may be mentioned:

N-(2-glycinamido-2-deoxy-β-deoxy-β-D-glucopyranosyl)-N-dodecyldodecanoylamide,

N-(2-glycinamido-2-deoxy-β-D-glucopyranosyl)-N-dodecyl-actadecanoylamide,

N-(2-glycinamido-2-deoxy-β-D-glucopyranosyl)-N-tetra-decyldodecanoylamide,

N-(2-L-alaninamido-2-deoxy-β-D-glucopyranosyl)-N-dodecyldodecanoylamide,

N-(2-D-alaninamido-2-deoxy-β-D-glucopyranosyl)-N-dodecyloctadecanoylamide,

N-(2-L-phenylalaninamido-2-deoxy-β-D-glucopyranosyl)-N-dodecyloctadecanoylamide,

N-(2-L-valinamido-2-deoxy-β-D-glucopyranosyl)-N-octa-decyldodecanoylamide,

N-(2-L-valinamido-2-deoxy-β-D-glucopyranosyl)-N-octa-decyltetradecanoylamide,

N-(2-L-leucinamido-2-deoxy-β-D-glucopyranosyl)-N-dodecyldodecanoylamide,

N-(2-L-leucinamido-2-deoxy-β-D-glucopyranosyl)-N-octa-decyldodecanoylamide (Bay R1005), and

N-(2-sarcosinamido-2-deoxy-β-D-glucopyranosyl)-N-octadecyldodecanoylamide.

Other adjuvants capable of promoting a Th1-type immune response (that is to say Th1 or Th1/Th2 type adjuvants) exist in the state of the art from which persons skilled in the art are capable of selecting the one which best corresponds to their needs. As a guide, there may be mentioned in particular liposomes; ISCOMS; microspheres; protein chochleates; vesicles consisting of nonionic surfactants; cationic amphiphilic dispersions in water; oil/water emulsions; muramidyldipeptide (MDP) and its derivatives such as glucosyl muramidyldipeptide (GMDP), threonyl-MDP, murametide and murapalmitin; as well as various other compounds such as monophosphoryl-lipid A (MPLA) major lipopolysaccharide from the wall of a bacterium, for example of *E. coli*, *Salmonella minnesota*, *Salmonella typhimurium* or *Shigella flexneri*; algan-glucan; gamma-inulin; calcitriol and loxoribine.

Useful liposomes for the purposes of the present invention may be selected in particular from pH-sensitive liposomes such as those formed by mixing cholesterol hemisuccinate (CHEMS) and dioleyl phosphatidyl ethanolamine (DOPE); liposomes containing cationic lipids recognized for their fusiogenic properties, such as 3-beta-(N-(N',N'-dimethylamino-ethane)carbamoyl)cholesterol (DC-chol) and its equivalents which are described in US Patent No. 5,283,185 and WO 96/14831, dimethyldioctadecylammonium bromide (DDAB) and the BAY compounds described in EP 91645 and EP 206 037, for example Bay R1005 (N-(2-deoxy-2-L-leucylamino-beta-D-glucopyranosyl)-N-octadecyldodecanoylamide acetate; and liposomes containing MTP-PE, a lipophilic derivative of MDP (muramidyldipeptide). These liposomes are useful for adding as adjuvant to all the immunogenic agents cited.

Useful ISCOMs for the purposes of the present invention may be selected in particular from those compounds of QuilA or of QS-21 combined with

cholesterol and optionally also with a phospholipid such as phosphatidylcholine. These are particularly advantageous for the formulation of the lipid-containing antigens.

Useful microspheres for the purposes of the present invention may be formed in particular from compounds such as polylactide-co-glycolide (PLAGA), alginate, chitosan, polyphosphazene and numerous other polymers.

Useful protein chochleates for the purposes of the present invention may be selected in particular from those formed from cholesterol and optionally an additional phospholipid such as phosphatidylcholine. These are especially advantageous for the formulation of the lipid-containing antigens.

Useful vesicles consisting of nonionic surfactants for the purposes of the present invention may be in particular formed by a mixture of 1-mono-palmitoyl glycerol, cholesterol and dicetylphosphate. They are an alternative to the conventional liposomes and may be used for the formulation of all the immunogenic agents cited.

Useful oil/water emulsions for the purposes of the present invention may be selected in particular from MF59 (Biocine-Chiron), SAF1 (Syntex) and the montanides ISA51 and ISA720 (Seppic).

The immunogenic agent derived from *Helicobacter* is advantageously selected from a preparation of inactivated *Helicobacter* bacteria, a *Helicobacter* cell lysate, a peptide and a polypeptide from *Helicobacter* in purified form.

For the purposes of the present invention, a preparation of inactivated bacteria may be obtained according to conventional methods well known to a person skilled in the art. Likewise for a bacterial lysate. A dose of inactivated bacteria or of cell lysate, appropriate for prophylactic or therapeutic purposes, can be determined by persons skilled in the art and depends on a number of factors such as the

individual for whom the vaccine is intended, *e.g.* age, the antigen itself, the route and mode of administration, the presence/absence or the type of adjuvant, as can be determined by persons skilled in the art. In general, it is indicated that an appropriate dose is from about 50 µg to 1 mg to about 1 mg of lysate.

A peptide or a polypeptide derived from *Helicobacter* may be purified from *Helicobacter* or obtained by genetic engineering techniques or alternatively by chemical synthesis. The latter process is advantageous in the case of peptides. "Peptide" is any amino acid chain whose size is less than about 50 amino acids. When the size is greater, the term "polypeptide", which is also interchangeable with the term "protein", is used. A useful peptide or polypeptide for the purposes of the present invention may be identical or similar to that which exists under natural conditions. It is similar in that it is capable of inducing an immune response of the same type but it may comprise certain structural variations such as, for example, a mutation, the addition of a residue of a lipid nature or alternatively it may be in fusion polypeptide or peptide form.

An appropriate dose of peptide or polypeptide for prophylactic or therapeutic purposes can be determined by persons skilled in the art and depends on a number of factors such as the individual for whom the vaccine is intended, *e.g.* age, the antigen itself, the route and mode of administration, the presence/absence or the type of adjuvant, as can be determined by persons skilled in the art. In general, it is indicated that an appropriate dose is from about 10 µg to about 1 mg, preferably at about 100 µg.

The immunogenic agent derived from *Helicobacter* may be any polypeptide from *Helicobacter*, *e.g. H. pylori*. This may be in particular a polypeptide present in the cytoplasm, a polypeptide of the inner or outer membrane or a polypeptide secreted in the external

medium. Numerous polypeptides from *Helicobacter* have already been described in the literature, either with reference to their amino acid sequence deduced from the sequence of the cloned or identified corresponding gene, or with reference to a purification process which makes it possible to obtain them in a form isolated from the rest of their natural environment. As a guide, the following documents may be mentioned in particular: WO 94/26901 and WO 96/34624 (HspA), WO 94/09023 (CagA), WO 96/38475 (HpaA), WO 93/181150 (cytotoxine), WO 95/27506 and Hazell et al., J. Gen. Microbiol. (1991) 137: 57 (catalase), FR 2 724 936 (membrane receptor for human lactoferrin), WO 96/41880 (AlpA), EP 752 473 (FibA) and O'Toole et al., J. Bact. (1991) 173: 505 (TsaA). Other polypeptides are also described in WO 96/40893, WO 96/33274, WO 96/25430 and WO 96/33220. A useful polypeptide for the purposes of the present invention may be identical or similar to one of those cited as a reference insofar as it is capable of promoting an immune response against *Helicobacter*. In order to meet this last condition, the immunogenic agent may also be a peptide derived from a polypeptide cited as a reference.

Advantageously, a polypeptide selected from the UreA and UreB subunits of *Helicobacter* urease is used (see WO 90/4030). Preferably, both are used, combined in urease apoenzyme form or alternatively in multimeric form (see WO 96/33732).

A useful pharmaceutical composition for the purposes of the present invention may contain a single immunogenic agent or several. For example, an advantageous composition may comprise UreA and UreB, *e.g.* in apoenzyme form, as well as one or more other polypeptides selected in particular from those mentioned above.

A useful pharmaceutical composition for the purposes of the present invention may, in addition, contain compounds other than the immunogenic agent itself and the Th1 or Th1/Th2 type adjuvant, the nature

of these compounds depending to some extent on the nature of the immunogenic agent, inactivated bacteria, cell lysate, peptide or polypeptide. For example, a composition may also comprise an adjuvant capable of promoting the induction of a Th2-type immune response, *e.g.* an aluminium compound such as aluminium hydroxide, aluminium phosphate or aluminium hydroxyphosphate. This may be advantageous insofar as the useful adjuvant for the purposes of the present invention is a Th1-type adjuvant such as QS-21.

The therapeutic or prophylactic efficacy of a method or of a use according to the invention may be evaluated according to standard methods, *e.g.* by measuring the induction of an immune response or the induction of a therapeutic or protective immunity using *e.g.* the mouse/*H. felis* model and the procedures described in Lee et al., Eur. J. Gastroenterology & Hepatology (1995) 7: 303 or Lee et al., J. Infect. Dis. (1995) 172: 161. Persons skilled in the art will realize that *H. felis* can be replaced in the mouse model by another *Helicobacter* species. For example, the efficacy of an immunogenic agent derived from *H. pylori* is preferably evaluated in a mouse model using an *H. pylori* strain adapted to mice. The efficacy may be determined by comparing the level of infection in the gastric tissue (by measuring the urease activity, the bacterial load or the condition of the gastritis) with that in a control group. A therapeutic effect or a protective effect exists when the infection is reduced compared with the control group.

A useful pharmaceutical composition for the purposes of the present invention may be manufactured in a conventional manner. In particular, it may be formulated with a pharmaceutically acceptable carrier or diluent, *e.g.* water or a saline solution. In general, the diluent or carrier may be selected according to the mode and route of administration and according to standard pharmaceutical practices. Appropriate carriers or diluents as well as what is

essential for the preparation of a pharmaceutical composition are described in *Remington's Pharmaceutical Sciences*, a standard reference book in this field.

The methods according to the invention as well as the compositions useful for these purposes may be used to treat or prevent *i.a. Helicobacter* infections and consequently the gastroduodenal diseases associated with these infections, including acute, chronic or atrophic gastritis, peptic ulcers, *e.g.* gastric or duodenal ulcers.

A pharmaceutical composition according to the invention may be administered conventionally, in particular by the mucosal route, *e.g.* by the ocular, oral, *e.g.* buccal or gastric, pulmonary, intestinal, rectal, vaginal or urinary route or by the systemic, in particular parenteral, *e.g.* intravenous, intramuscular, intradermal, intraepidermal and subcutaneous, route. Preferably, the parenteral route is used. When the parenteral route is used, a site of administration situated under the diaphragm of an individual is preferably chosen. The dorsolumbar region constitutes, for example, an appropriate site of administration, in particular for the intraepidermal, intramuscular, intradermal and subcutaneous routes, these latter routes being chosen in preference to the intravenous route.

To obtain a protective or therapeutic effect, the operation which consists in administering a useful pharmaceutical composition for the purposes of the present invention may be repeated once or several times, preferably at least twice, leaving a certain time interval between each administration, which interval is of the order of a week or a month. Its precise determination is within the capability of persons skilled in the art and may vary according to various factors such as the nature of the immunogenic agent, the age of the individual and the like.

According to a specific mode, the vaccination procedure is carried out using the same route of

administration during the first immunization and the boosters. In this particular case, the administration is said to be for example of the strict systemic type.

"A method in which the administration of the immunogenic agent is carried out by the strict systemic route" is defined as a method not using a route of administration other than the systemic route. For example, a method in which the immunogenic agent is administered by the systemic route and by the mucosal route does not correspond to the definition given above. In other words, "a method in which the administration of the immunogenic agent is carried out by the strict systemic route" should be understood to mean a method in which the immunogenic agent is administered by the systemic route excluding any other route, in particular the mucosal route.

By way of a nonlimiting illustration, there may be mentioned a vaccination scheme which consists in administering the urease apoenzyme in combination with QS-21, DC-chol or one of their equivalents, three times by the subcutaneous route, in the dorsolumbar region with an interval of two to four weeks between each administration.

It is also possible to predict that the administration of a pharmaceutical composition according to the present invention may be a single step forming part of a more elaborate vaccination procedure. For example, a pharmaceutical composition according to the present invention may be preceded or followed by the administration of a pharmaceutical composition containing an immunogenic agent derived from *Helicobacter* chosen independently from those stated above or among others such as a vaccinal vector or a DNA molecule, but not containing QS-21, DC-chol or one of their equivalents, it being possible for the latter to then be replaced by a completely different adjuvant, it being possible for the two compositions to be administered by identical or different routes.

By way of a nonlimiting illustration, the following procedures may be mentioned:

- A first immunization by the systemic route, with the urease apoenzyme in the presence of QS-21, followed by two boosters with the urease apoenzyme in the presence of QS-21 or LT by the mucosal route; and

- A first immunization by the systemic route, with a poxvirus encoding UreA and UreB followed by two boosters with the urease apoenzyme in the presence of QS-21, by the systemic or mucosal route.

Immunogenic agents, other than those described above and which are capable of being used in a multistep vaccination procedure comprising a step of administration using a useful medicament for the purposes of the present invention or a composition according to the present invention, may be selected from a polynucleotide molecule, in particular a DNA molecule comprising a sequence encoding a peptide or a polypeptide from *Helicobacter* placed under the control of the elements necessary for its expression in a mammalian cell; or alternatively a vaccinal vector comprising a sequence encoding a peptide or a polypeptide from *Helicobacter* placed under the control of the elements necessary for its expression in a mammalian cell (if this is a viral vector) or in a prokaryote (if this is a bacterial vector).

The DNA molecule may advantageously be a plasmid which is incapable both of replicating and of substantially integrating into the genome of a mammal. The abovementioned coding sequence is placed under the control of a promoter allowing expression in a mammalian cell. This promoter may be ubiquitous or specific for a tissue. Among the ubiquitous promoters, there may be mentioned the cytomegalovirus early promoter (described in US Patent No. 4,168,062) and the Rous sarcoma virus promoter (described in Norton & Coffin, Molec. Cell. Biol. (1985) 5: 281). The desmin promoter (Li et al., Gene (1989) 78: 244443; Li & Paulin, J. Biol. Chem. (1993) 268: 10403) which is a

selective promoter allows expression in muscle cells and also in skin cells. A promoter specific for the muscle cells is for example the promoter of the myosin or dystrophin gene. Plasmid vectors which can be used for the purposes of the present invention are described *i.a.* in WO 94/21797 and Hartikka et al., Human Gene Therapy (1996) 7: 1205.

In a useful pharmaceutical composition for the purposes of the present invention, the nucleotide molecule, *e.g.* the DNA molecule, may be formulated or otherwise. The choice of formulation is highly varied. The DNA may be simply diluted in a physiologically acceptable solution with or without carrier. When the latter is present, it may be isotonic or weakly hypertonic and may have a low ionic strength. For example, these conditions may be fulfilled by a sucrose solution, *e.g.* at 20%.

Alternatively, the polynucleotide may be combined with agents which promote entry into the cell. This may be (i) a chemical agent which modifies cell permeability, such as bupivacaine (see for example WO 94/16737) or (ii) an agent which is combined with the polynucleotide and which acts as a vehicle facilitating the transport of the polynucleotide. The latter may be in particular cationic polymers, *e.g.* polylysine or a polyamine, *e.g.* derivatives of spermine (see WO 93/18759). This may also be fusogenic peptides, *e.g.* GALA or Gramicidin S (see WO 93/19768) or alternatively peptides derived from viral fusion proteins.

This may also be anionic or cationic lipids. The anionic or neutral lipids have been known for a long time to be capable of serving as transporting agents, for example in the form of liposomes, for a large number of compounds including polynucleotides. A detailed description of these liposomes, of their constituents and of the processes for their manufacture is for example provided by Liposomes: A Practical Approach, RPC New Ed., IRL press (1990).

The cationic lipids are also known and are commonly used as transporting agents for polynucleotides. There may be mentioned for example Lipofectin™ also known by the name DOTMA (N-[1-(2,3-dioleyloxy) propyl]-N,N,N-trimethylammonium chloride), DOTAP (1,2-bis(oleyloxy)-3-(trimethyl-ammonio)propane), DDAB (dimethyldioctadecylammonium bromide), DOGS (dioctadecylamidoglycyl spermine) and cholesterol derivatives such as DC-chol (3-beta-(N-(N',N'-dimethylaminoethane)carbamoyl)cholesterol). A description of these lipids is provided by EP 187,702, WO 90/11092, US Patent No. 5,283,185, WO 91/15501, WO 95/26356 and US Patent No. 5,527,928. The cationic lipids are preferably used with a neutral lipid such as DOPE (dioleylphosphatidylethanolamine) as is for example described in WO 90/11092.

Gold or tungsten microparticles may also be used as transporting agents, as described in WO 91/359, WO 93/17706 and Tang et al., Nature (1992) 356: 152. In this particular case, the polynucleotide is precipitated on the microparticles in the presence of calcium chloride and spermidine and then the whole is administered by a high-speed jet into the dermis or into the epidermis using an apparatus with no needle such as those described in US Patents No. 4,945,050 and No. 5,015,580 and WO 94/24243.

The quantity of DNA which may be used to vaccinate an individual depends on a number of factors such as for example the strength of the promoter used to express the antigen, the immunogenicity of the product expressed, the condition of the mammal for whom the administration is intended (*e.g.* the weight, age and general state of health), the mode of administration and the type of formulation. In general, an appropriate dose for prophylactic or therapeutic use in an adult of the human species is from about 1 µg to about 5 mg, preferably from about 10 µg to about 1 mg, most preferably from about 25 µg to about 500 µg.

Vaccinal vectors are among the immunogenic agents mentioned above. Adenoviruses and poxviruses in particular are among the vectors of viral origin. An example of a vector derived from an adenovirus as well as a method for constructing a vector capable of expressing a DNA molecule encoding a useful peptide or polypeptide for the purposes of the present invention are described in US Patent No. 4,920,209. Poxviruses which may be used likewise are for example the vaccinia and canarypox viruses. They are described respectively in US Patents No. 4,722,848 and 5,364,773 (see also *e.g.* Tartaglia et al., Virology (1992) 188: 217 and Taylor et al., Vaccine (1995) 13: 539). Poxviruses capable of expressing a useful peptide or polypeptide for the purposes of the present invention may be obtained by homologous recombination as described in Kieny et al., Nature (1984) 312: 163, such that the DNA fragment encoding the peptide or polypeptide is placed under conditions appropriate for its expression in mammalian cells. A bacterial vector such as the bile Calmette-Guérin bacillus may also be envisaged.

In general, the dose of a viral vector intended for prophylactic or therapeutic purposes may be from about 1×10^4 to about 1×10^{11}, advantageously from about 1×10^7 to about 1×10^{10}, preferably from about 1×10^7 to about 1×10^9 plaque forming units *per* kilogram.

Among the bacterial vectors, there may be mentioned *Shigella*, *Salmonella*, *Vibrio cholerae*, *Lactobacillus* and *Streptococcus*. Nontoxic mutant strains of *Vibrio cholerae* which may be useful as live vaccine are described in Mekalanos et al., Nature (1983) 306: 551 and US Patent No. 4,882,278 (strain in which a substantial part of the region encoding each of the two alleles *ctxA* has been deleted so that no functional toxin can be produced); WO 92/11354 (strain in which the *irgA* locus is inactivated by mutation; this mutation may be combined in the same strain with *ctxA* mutations); and WO 94/1533 (mutant obtained by

deletion lacking functional *ctxA* and *attRS1* sequences). These strains may be modified genetically in order to express heterologous antigens as described in WO 94/19482.

Attenuated strains of *Salmonella typhimurium*, genetically modified or otherwise for the recombinant expression of heterologous antigens, as well as their use as vaccines are described in Nakayama et al., BioTechnology (1988) 6: 693 and WO 92/11361.

Other bacteria useful as vaccinal vectors are described in High et al., EMBO (1992) 11: 1991 and Sizemore et al., Science (1995) 270: 299 (*Shigella flexneri*); Medaglini et al., Proc. Natl. Acad. Sci. USA (1995) 92: 6868 (*Streptococcus gordonii*); and Flynn J.L., Cell. Mol. Biol. (1994) 40 (suppl. I): 31, WO 88/6626, WO 90/0594, WO 91/13157, WO 92/1796 and WO 92/21376 (Calmette-Guérin bacillus).

In bacterial vectors, the DNA sequence encoding a peptide or polypeptide from *Helicobacter* may be inserted into the bacterial genome or alternatively remain in the free state, carried by a plasmid.

Likewise, a DNA molecule or a vaccinal vector may comprise a sequence encoding any polypeptide or peptide described above.

A DNA molecule, preferably a viral vaccinal vector, may also comprise a sequence encoding a cytokine, for example a lymphokine such as interleukin-2 or -12, under the control of elements appropriate for expression in a mammalian cell. An alternative to this option also consists in adding to a useful pharmaceutical composition for the purposes of the present invention comprising a DNA molecule or a vector, another molecule or viral vector encoding a cytokine.

In general, the subject of the invention is therefore also a pharmaceutical composition intended to treat or prevent a *Helicobacter* infection which comprises, for consecutive administration: (i) a first product containing (a) an immunogenic agent derived

from *Helicobacter* selected independently from a preparation of inactivated *Helicobacter* bacteria, a Helicobacter cell lysate, a peptide and a polypeptide from *Helicobacter* in purified form, and (b) a compound capable of promoting the induction of a Th1-type immune response and (ii) a second product containing an immunogenic agent derived from *Helicobacter* selected independently from a preparation of inactivated *Helicobacter* bacteria, a *Helicobacter* cell lysate, a peptide and a polypeptide from *Helicobacter* in purified form, a DNA molecule comprising a sequence encoding a peptide or a polypeptide from *Helicobacter* placed under the control of the elements necessary for its expression and a vaccinal vector comprising a sequence encoding a peptide or a polypeptide from *Helicobacter* placed under the control of the elements necessary for its expression, preferably provided that when a first product contains a peptide or a polypeptide and a second product contains a DNA molecule or a vaccinal vector, the said coding sequence of the DNA molecule or of the vaccinal vector encodes the peptide or polypeptide contained in the first product.

In the description above, reference was made essentially to *Helicobacter* infections and to the means for combating them by way of prevention and prophylaxis. However, it should be understood that the principles and methods stated above can be applied *mutatis mutandis* to any other infection induced by any microorganism whose seat is the stomach, the duodenum or the intestine.

It is specified, in addition, that all the documents published and cited in the present application are incorporated by reference.

The invention is illustrated below with reference to the following figures.

Figure 1 refers to Example 1 and presents the levels of urease activity after a challenge, measured 4 h after sacrificing mice which have received 3 times, on D0, D28 and D56: (a) a urease preparation

encapsulated at about 80% in DC-chol liposomes, in the dorsolumbar muscles; or (b) a urease preparation with cholera toxin adjuvant, by the intragastric route. Experiments (c) and (d) correspond respectively to the positive and negative controls.

Figure 2 refers to Example 1 and presents the levels of urease activity after a challenge, measured 4 h after sacrificing mice having received 3 times, on D0, D28 and D56: (a) a urease preparation with cholera toxin adjuvant, by the intragastric route or (b) a urease preparation with PCPP adjuvant, by the subcutaneous route in the left posterior sublumbar part; or (c) a urease preparation with QS-21 adjuvant, by the subcutaneous route in the lower back. Experiments (c) and (d) correspond respectively to the positive and negative controls.

Figure 3 presents the quantities of serum immunoglobulins induced in monkeys subjected to the immunization procedures described in Example 2, and expressed as ELISA titre. A control group comprising 4 monkeys and three test groups are formed, each of the test groups comprising 8 monkeys; each test group is divided into two subgroups of 4 monkeys, one receiving only the inactivated *H. pylori* preparation (1, 2 and 3) and the other receiving the inactivated *H. pylori* preparation and recombinant urease (1u, 2u and 3u). Group 1 and 1u corresponds to the administration procedure [nasal + intragastric, 4 times]; group 2 and 2u corresponds to the administration procedure [intramuscular, 4 times]; group 1 and 1u corresponds to the administration procedure [nasal + intragastric, intramuscular, nasal + intragastric, intramuscular]. The ELISA titre is measured three times: a first time at D0 (white band), a second time at D42 (shaded band), a third time at D78 (black band).

Figure 4 presents the quantities of serum immunoglobulins induced in mice subjected to the immunization procedures described in Example 3, and expressed as ELISA titre. O indicates the ELISA IgG2a

titre and ◆ indicates the ELISA IgG1 titre. Two control groups (positive and negative controls), four test groups (A1 to A4) as well as a reference group (LT) are formed, each of the groups comprising 10 mice. The measurements of the quantities of serum immunoglobulins are carried out for only 5 mice among the ten. The mice of the A1 to A4 groups received 10 µg doses of urease by the subcutaneous route in the left posterior sublumbar part, in the presence of QS-21 (A1), Bay R1005 (A2), DC-chol (A3) or PCPP (A4). The mice of the reference group received 40 µg doses of urease by the oral route in the presence of *E. coli* heat-labile protein.

Figure 5 presents the levels of urease activity measured at the level of the stomachic mucous membrane, at OD_{550} 4 hours after the mice subjected to the immunization procedures described in Example 3 have been sacrificed. The groups are as described in Figure 4.

Figure 6 presents the levels of urease activity measured at the level of the stomachic mucous membrane at OD_{550} 24 hours after the mice subjected to the immunization procedures described in Example 3 have been sacrificed. The groups are as described in Figure 4.

Figure 7 presents the bacterial load measured at the level of the stomachic mucosal membrane after the mice subjected to the immunization procedures described in Example 3 have been sacrificed. The groups are as described in Figure 4.

Example 1: Immunization studies in mice

1A- Materials and methods

Mice

6/8-week old female Swiss mice were provided by Janvier (France). During the whole experiment, sterilized materials were used; the cages were

protected by "isocaps"; the mice were fed with filtered water and irradiated food.

Administration procedure

During each experiment, the mice received 3 doses of the same product; each dose at 28-day intervals (days 0, 28 and 56). The administration of the product was carried out by the nasal route (up to 50 µl on waking mice), by the oral route (300 µl in 0.2 M $NaHCO_3$ by gastric gavage), or by the subcutaneous route (300 µl under the skin of the neck or under the skin on the left side of the lumbar region). In some cases, an intramuscular inoculation was carried out (50 µl) in the dorsolumbar muscles of anaesthetized mice. 10 µg of urease were administered by the nasal, subcutaneous or intramuscular route, and 40 µg by the oral route. As regards the inactivated bacterial preparation, 400 µg of cells were administered by the subcutaneous route or by the oral route.

Antigens and adjuvants

The *H. pylori* urease apoenzyme was expressed in *E. coli* and purified as has been described in Example 5 of WO 96/31235. In the remainder of the text, the simple term of urease is used to designate this apoenzyme.

DC-chol liposomes containing urease are prepared as follows: first of all, to obtain a dry lipid film containing 100 mg of DC-chol (R-Gene Therapeutics) and 100 mg of DOPC (dioleylphosphatidylcholine) (Avanti Polar Lipids), these products are mixed in powdered form in about 5 ml of chloroform. The solution is allowed to evaporate under vacuum using a rotary evaporator. The film thus obtained on the walls of the container is dried under high vacuum for at least 4 h. In parallel, 20 mg of a urease lyophilisate and 100 mg of sucrose are diluted in 13.33 ml of 20 mM Hepes buffer pH 7.2. Ten ml of this preparation (which contains 1.5 mg of urease and

0.75% sucrose) is filtered on the 0.220 µm Millex filter and then used to rehydrate the lipid film. The suspension is stirred for 4 h and then either extruded (10 passes on a 0.2 µm polycarbonate membrane) or microfluidized (10 passes at a pressure of 500 kPa in a Microfluidics Co Y10 microfluidizer). In the liposome suspension thus obtained, the level of encapsulated urease is from 10 to 60%. This suspension is lyophilized after having adjusted the sucrose concentration to 5% (425 mg of sucrose are added per 10 ml). Before use, the lyophilisate is taken up in an appropriate volume of water or buffer and the suspension is purified on a discontinuous sucrose gradient (steps of 0, 30 and 60%) so as to obtain a preparation in which the quantity of encapsulated urease is greater than about 70% compared with the total quantity of urease.

The cholera toxin is used as mucosal adjuvant in an amount of 10 µg/dose of urease or of bacterial preparation.

The QS-21 (Cambridge Biosciences) is used as adjuvant in an amount of 15 µg/dose of urease.

The polyphosphazene (PCPP) (Virus Research Institute) is used as adjuvant in an amount of 100 µg/dose of urease.

Challenge

Two weeks after the second booster, the mice were subjected to a gastric gavage with 300 µl of a suspension of a strain of *H. pylori* adapted to the mice, the strain ORV2002 (1×10^7 live bacteria in 200 µl of PBS; OD_{550} of about 0.5). One group which received no dose of antigen and which serves as control is challenged likewise.

Analysis of the challenge

Four weeks after the challenge, the mice were sacrificed by breaking the cervical vertebrae. The stomachs were removed in order to evaluate the urease

activity and to make histological analyses. The urease activity was evaluated after 4 and 24 hours (OD at 550 nm) with the Jatrox test, Procter & Gamble) and after 24 hours the number of mice still negative (OD less than 0.1) was noted.

Measurement of the local antibody response by ELISPOT (salivary glands and stomach).

The ELISPOTs were performed in accordance with Mega et al., J. Immunol. (1992) 148: 2030. The plates were coated with an extract of *H. pylori* proteins at a concentration of 50 µg/ml.

To test the antibody response at the level of the stomach, we modified the method as follows: half of the stomach was cut into 1-mm^2 pieces with an automatic apparatus for cutting human tissues (McIllwain Laboratories, Gilford, UK) and the digestion carried out with Dispase (2 mg/ml, Boeringher Mannheim) in 2 ml of a modified Joklil solution to which 10% horse serum (Gibco), glutamine and antibiotics were added. Four half-hour digestions were performed at 37°C with gentle mixing. The cells thus digested were filtered after each step using 70 µm filters (Falcon), and then washed 3 times in a solution of RPMI 1640 (Gibco) supplemented with 5% foetal calf serum (FCS), and incubated in the same solution for at least 4 hours in plates covered with nitrocellulose (Millipore) (100 µl/well, 4 wells). Between 1 and 3 × 10^5 cells are obtained per half stomach (the cells of large size and the macrophages were not counted).

The biotinylated IgA and the streptavidin-biotinylated peroxidase complex were obtained from Amersham. The spots were revealed under the action of the AEC substrate (Sigma) and as soon as the plates are dry, they were counted under a microscope (magnification ×16 or ×40). The mean values corresponding to the number of IgA spots in four wells were calculated and expressed as the number of spots/10^6 cells.

Analysis of the response by ELISA

The analyses by ELISA were performed in accordance with the standard procedure (the biotinylated conjugates and the streptavidin-peroxidase were obtained from Amersham and the OPD (O-phenyl-diamine dihydrochloride) substrate from Sigma). The plates were coated with *H. pylori* extracts (5 µg/ml) in carbonate buffer. A control serum from mice directed against the *H. pylori* extract was introduced in each experiment. The titre corresponds to the reciprocal of the dilution giving an OD of 1.5 at 490 nm.

1B- Results

The results are presented in Figures 1 and 2 described above and by the following comments:

Before any comments on the subject of Figures 1 and 2, it should be noted that these figures present the results obtained with the antigen used with the cholera toxin adjuvant and administered by the intragastric route. This experiment is termed standard reference experiment since the prior art CT/IG combination is that which gives the best results up until now.

Figure 1 shows that a urease preparation encapsulated into DC-chol liposomes gives results as good as those obtained in the standard reference experiment.

Furthermore, reference can be made to experiments (a) to (d) whose results in terms of urease activity 4 h after the mice have been sacrificed are reported in Figure 1 and it is indicated that the number of mice which are still negative for the urease activity 24 h after having been sacrificed is respectively (a) 5/10, (b) 4/10, (c) 0/10 and (d) 10/10. This is in agreement with what was previously concluded in the paragraph; namely that experiment (a) leads to results similar to those obtained during the standard reference experiment.

Figure 2 shows that a urease preparation with QS-21 adjuvant gives results as good as those obtained in the standard reference experiment. Furthermore, this figure shows that the results which are obtained using PCPP as adjuvant are a lot less satisfactory than those obtained with QS-21. This can be explained since PCPP induces, preferentially with urease, a Th2-type response whereas QS-21 with urease induces a Th1/Th2 balanced response, as demonstrated in the table below.

Furthermore, reference can be made to experiments (a) to (e) whose results in terms of urease activity 4 h after the mice have been sacrificed are reported in Figure 2 and it is indicated that the number of mice which are still negative for the urease activity 24 h after having been sacrificed is respectively (a) 1/8, (b) 0/8, (c) 5/8, (d) 0/8 and (e) 10/10. This is in agreement with what was previously concluded in the paragraph, namely that experiment (c) leads to results similar to those obtained during the standard reference experiment.

The table below presents the quantities of serum IgA, IgG1 and IgG2a induced during experiments whose results in terms of urease activity are reported in Figures 1 and 2 as well as the number of mice whose urease activity is characterized by an OD of less than 0.1 after 4 and 24 h after sacrifice. The quantities of IgA, IgG1 and IgG2a are expressed as ELISA titre.

	urease CT IG	urease DC-chol SC	urease PCPP SC	urease QS21 SC
IgA	45	0	58	1
IgG1	65700	620000	2930520	2970399
IgG2a	20200	321000	26200	1136095
OD<0.14 h	5/10	5/10	0/8	6/8
OD<0.124 h	4/10	5/10	0/8	5/8

The results presented in the table above show that when the subcutaneous route is used (as well as an adjuvant appropriate for this route), the serum

antibody level is high, which is not the case after using the intragastric route (and adjuvant which is appropriate for this route). Furthermore, these results show that when DC-chol or QS-21 is used, a high IgG2a level is obtained comparable to the IgG1 level in order of magnitude. This indicates that these adjuvants have the capacity to induce not only a Th2 response, but also a Th1 response. On the other hand, when PCPP is used, the IgG2a level obtained is substantially lower than the IgG1 level. It can be concluded that the latter adjuvant induces essentially a Th2 response and cannot therefore be a useful adjuvant for the purposes of the present invention.

Example 2: Immunization studies in monkeys

2A- Materials and methods

Monkeys

Twenty eight 2-year old monkeys (*Macaca fascicularis*) obtained from Mauritius were used in this study. Before subjecting the monkeys to the various immunization procedures described below, a biopsy showed that most of them were chronically infected with organisms similar to *Gastrospirillum hominis* (GHLO) or *H. heilmanii*.

Administration procedures

Since nearly all the monkeys were infected with GHLOs, it was decided to test the efficacy of the various procedures in therapy. Three procedures were used, as summarized in the table below:

Group	D0	D21	D42	D63
1 and 1u	IN + IG	IN + IG	IN + IG	IN + IG
2 and 2u	IM	IM	IM	IM
3 and 3u	IM	IN + IG	IM	IN + IG

It is specified that the administration by the intramuscular route was carried out in the dorsolumbar muscles.

Antigens and adjuvants

Since there is a cross-reactivity between the GPLOs and *H. pylori*, it was chosen to use a preparation of inactivated *H. pylori* bacteria, as described in Example 1A, alone or in combination with recombinant urease prepared according to the method referenced in Example 1A.

The *E. coli* heat-labile toxin (LT) (Sigma) or the B subunit of the cholera toxin (CTB) (Pasteur Mérieux sérums & vaccins) was used as mucosal adjuvant whereas DC-chol was used as parenteral adjuvant. DC-chol powder is simply rehydrated with an antigen preparation.

The doses used are as follows:

Route	Microorganisms	Urease	DC-chol	LT	CTB
IG	400 µg	2.5 mg	-	25 µg	-
IN	400 µg	400 µg	-	25 ng	25 µg
IM	400 µg	100 µg	400 µg	-	-

Biopsies, urease test and bacteriological/histological study

A biopsy was performed on each of the monkeys before and after immunization (one month after the third booster). Using the biopsies, a urease test and a histological study were performed.

The urease activity is evaluated using the Jatrox kit (Procter & Gamble). The level of this activity is estimated as follows, in a decreasing manner: level 3, pink colour appearing during the first 10 minutes; level 2, pink colour appearing between 10 and 30 minutes after the addition of the reagents; level 1, pink colour appearing between 30 min and 4 h and level 0, weak or no colour after 4 h.

The histological studies were performed using biopsies fixed in formalin and the bacterial load was quantified as follows: absence of bacteria (0); a few bacteria of the *Helicobacter* type (0.5); fairly numerous bacteria (1); numerous bacteria (2); highly numerous bacteria (3). A difference of one level (for example from 1 to 2) corresponds to a number of bacteria 5 times greater.

Analysis of the response by the ELISA test

An ELISA test is carried out as described in Example 1A.

1B- Results

The table below relates to the bacterial load which, before and after immunization, is assessed using two tests: (i) by evaluating the urease activity and (ii) by carrying out a histological study. The results relating thereto are presented in columns 3 to 6. The last three columns indicate for each group (control, 1, 2 or 3) the number of monkeys for which the bacterial load remains unchanged after immunization (→) according to the two tests; or appears lower (↘) or increased (↗) in at least one of the two tests, the other test indicating a stationary bacterial load. When the results of the two tests show a similar variation, the upwards or downwards arrow is double.

Monkeys	Group	Urease activity before immunization	Urease activity after immunization	Histology before immunization	Histology after immunization	Variation ↘	Variation →	Variation ↗
H 282	C	2-2	3-2	2	3-2			
J 005	C	2-2	2-1	2	1-0	1/4	1/4	2/4
J 852	C	0-0	2-0	0	1-1			(2/4↗↗)
J 476	C	0-0	2-0	0	1-1			
H 799	1	2-2	2-2	2	2-2			
J 845	1	2-2	3-2	2	2-1			
J 205	1	1-1	2-2	0	1			
J 328	1	2-2	1-2	3	3-2	1/8	5/8	2/8
J 197	1u	2-2	3-2	2	3			(1/8↗↗)
H 025	1u	2-2	2-2	1	1-1			
G 460	1u	2-2	3-2	3	2-3			
J 607	1u	2-2	2-2	2	2			
H 549	2	3-3	2-2	3	2-3			
H 622	2	3-3	1-1	2	2-3			
H 504	2	3-3	1-1	2	2-1			
H 798	2	1-1	0-1	1	1-1			
J 367	2u	2-2	2-1	3	2-3	6/8	1/8	1/8
G 486	2u	2-2	2-2	1	2-2			
J 522	2u	2-2	0-0	2	2-2			
G 722	2u	3-3	2-0	2	2-3			
H 820	3	3-3	2-2	3	2-2			
J 557	3	2-2	1-0	2	1-2*			
H 588	3	2-2	2-0	3	1-2			
J 153	3	3-3	3-3	2	3-3	5/8	0	3/8
H 480	3u	2-2	2-2	2	3-3	(3/8↘↘)		
J 344	3u	3-3	2-0	3	2-2			
H 710	3u	2-2	2-2	2	3-3			
J 262	3u	3-3	2-2	3	3-2			

Thus, this table reveals that in the group having been subjected to an immunization procedure by the strict mucosal route, the results are substantially identical to those obtained with the negative control

group. On the other hand, in the groups having been subjected to an immunization procedure by the mixed mucosal and intramuscular route or by the strict intramuscular route, a marked reduction in the bacterial load is observed. This highlights the importance of the immunization conditions and in particular of the adjuvant used; consequently, the use of an adjuvant such as DC-chol, capable of promoting a balanced Th1 and Th2 response, is recommended in order to obtain a protective effect.

These results are to be placed in perspective with other results relating to the serum antibody levels which are presented in Figure 3. This figure shows that the immunization scheme by the strict mucosal route (1 and 1u) leads to results which are very similar to those of the negative control group. On the other hand, the immunization scheme by the mixed mucosal and intramuscular route (2 and 2u), and better still the immunization scheme by the strict intramuscular route (3 and 3u), makes it possible to induce antibody levels substantially greater than those of the control group.

Thus, a high serum response may be correlated with a protective effect, whereas *a contrario*, a low response is linked to the absence of a protective effect. The immunization conditions which make it possible to obtain the desired effect (high serum response and protective effect) include the use of the parenteral route targeted in the subdiaphragmatic region or that of a Th1 adjuvant.

Example 3: Other immunization studies in mice

3A- Materials and methods

Mice

6/8-week old female Swiss mice were provided by Janvier (France). During the whole experiment, sterilized materials were used; the cages were

protected by "isocaps"; the mice were fed with filtered water and irradiated food.

Administration procedure

During each experiment, the mice received 3 doses of the same product; each dose at 21-day intervals (days 0, 21 and 42). The administration of the product was carried out by the oral route (300 µl in 0.2 M $NaHCO_3$ by gastric gavage), or by the subcutaneous route (300 µl under the skin on the left side of the lumbar region). Ten µg of urease were administered subcutaneously and 40 µg by the oral route.

Antigens and adjuvants

The *H. pylori* urease apoenzyme was expressed in *E. coli* and purified as has been described in Example 5 of WO 96/31235. In the remainder of the text, the simple term of urease is used to designate this apoenzyme.

The *E. coli* heat-labile toxin (Sigma) is used as mucosal adjuvant in an amount of 1 µg/dose of urease.

QS-21 (Cambridge Biosciences) is used as adjuvant in an amount of 15 µg/dose of urease.

Bay R1005 (Bayer) is used as adjuvant in an amount of 400 µg/dose of urease.

DC-chol (R-Gene Therapeutics) is used as adjuvant in an amount of 65 µg/dose of urease.

The polyphosphazene (PCPP) (Virus Research Institute) is used as adjuvant in an amount of 100 µg/dose of urease.

Challenge

Four weeks after the second booster, the mice were subjected to a gastric gavage with 300 µl (3×10^6 live bacteria) of a suspension of a strain of *H. pylori* adapted to the mice and resistant to Streptomycin, the strain ORV2001. One group which received no dose of

antigen and which serves as control is challenged likewise.

The challenge suspension is prepared as follows: *H. pylori* is cultured on Muller-Hinton agar (Difco) containing 5% sheep blood (bioMérieux) (MHA medium) which contains the following antibiotics from Sigma: Trimethoprim 5 µg/ml, Vancomycin 10 µg/ml, Polymixin B 1.3 µg/ml, Amphotericin 5 µg/ml and Streptomycin 50 µg/ml. The culture dishes are incubated for 3 days at 37°C under microaerophilic conditions (Anaerocult C, Merck). This culture is harvested in order to inoculate a 75 cm^2 flask provided with vents (Costar) containing 50 ml of Brucella broth supplemented with 5% foetal calf serum and with the abovementioned antibiotics. The flask is incubated under microaerophilic conditions, with gentle shaking for 24 hours. The suspension is then diluted in Brucella broth in order to give an OD of 0.1 at 550 nm (that is to say 10^7 CFU/ml).

Analysis of the challenge

Four weeks after the challenge, the mice were sacrificed by breaking the cervical vertebrae. The stomachs were removed in order to evaluate the urease activity and the bacterial load by quantitative culture. A longitudinal quarter of the stomach (antrum + corpus) is used for each of the tests. The urease activity was evaluated after 4 and 24 hours (OD at 550 nm) with the Jatrox test, Procter & Gamble) and after 24 hours the number of mice still negative (OD less than 0.1) was noted.

Evaluation of the infection by quantitative culture of *H. pylori*

At the time when the mice are sacrificed, the mucous membrane of a quarter of the stomach of each mouse is placed in the Portagem medium from bioMérieux and then within the next two hours, transferred into a culture chamber. The sample is then homogenized using a

Dounce homogenizer (Wheaton, Millville USA) containing 1 ml of Brucella medium (Brucella broth) and serially diluted up to 10^{-3}. One hundred µl of each dilution (10^{0}, 10^{-1}, 10^{-2} and 10^{-3}) are spread in Petri dishes containing MHA medium supplemented with the abovementioned antibiotics, for culturing at 37°C under microaerophilic conditions for 4 or 5 days. The number of viable bacteria is then counted. *H. pylori* is identified by its morphology revealed by Gram staining and by positive reactions to urease, catalase and oxidase tests.

Analysis of the response by ELISA

The analyses by ELISA were performed in accordance with the standard procedure (the biotinylated conjugates and the streptavidin-peroxidase were obtained from Amersham and the OPD substrate from Sigma). The plates were coated with *H. pylori* extracts (5 µg/ml) in carbonate buffer. A control serum from mice directed against the *H. pylori* extract was introduced in each experiment. The titre corresponds to the reciprocal of the dilution giving an OD of 1.5 at 490 nm.

3B- Results

Before any comments on the subject of Figures 4 to 7, it should be noted that these figures present the results obtained with the antigen used with the LT adjuvant and administered by the intragastric route. This experiment is termed standard reference experiments since the prior art LT/IG combination is that which gives the best results up until now.

Serum response

As shown in Figure 4, after three immunizations, all the mice immunized by the subcutaneous route have a high serum IgG response. On the basis of the IgG1:IgG2a ratios, it can be noted that PCPP induces a predominant response of the Th2

type (high IgG1 level, low IgG2a level). Bay R1005 and DC-chol induce a more balanced response of the Th1/Th2 type. Finally, QS-21 induces a predominant response of the Th1 type. In fact, the main difference between the four groups of mice A1 to A4 lies in their IgG2a titres, the IgG1 titres all being similar.

Protection after challenge

Figures 5 to 7 show that the level of protection in groups A1 and A2 is similar to or even better than that observed in the reference group (LT). They are groups which received doses of urease in the presence of QS-21 and of Bay R1005 respectively. Group A3 (DC-chol) shows a slightly lower level of protection. On the other hand, in group A4 (PCPP), it is not possible to demonstrate a high protective effect. It should be noted that the results presented in Figures 5 to 7 are consistent with each other.

When the results presented in Figure 4, on the one hand, and Figures 5 to 7, on the other, are compared, it can be rightly concluded that the use of an adjuvant capable of inducing a Th1 or Th1/Th2 response (QS-21, Bay R1005 or DC-chol) promotes the coming into play of a protective effect, contrary to the use of a Th2-type adjuvant (PCPP).

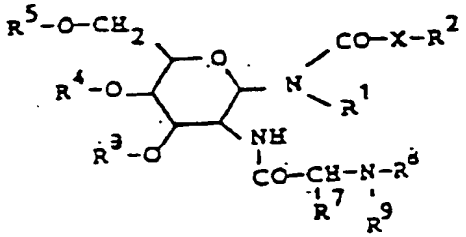
Claims

1. Pharmaceutical composition which comprises an immunogenic agent derived from *Helicobacter* and at least one compound selected from:

(i) saponins purified from an extract of *Quillaja saponaria*;

(ii) cationic lipids which are weak inhibitors of protein kinase C and have a structure which includes a lipophilic group derived from cholesterol, a bonding group selected from carboxyamides and carbamoyls, a spacer arm consisting of a branched or unbranched linear alkyl chain of 1 to 20 carbon atoms, and a cationic amine group selected from primary, secondary, tertiary and quaternary amines; and

(iii) glycolipopeptides of formula (I):



(I)

in which

R^1 represents an alkyl residue saturated or unsaturated once or several times and comprising from 1 to 50 carbon atoms,

X represents $-CH_2-$, $-O-$ or $-NH-$,

R^2 represents a hydrogen atom or an alkyl residue saturated or unsaturated once or several times and comprising from 1 to 50 carbon atoms,

R^3, R^4 and R^5 each represent, independently of each other, a hydrogen atom or an acyl-$CO-R^6$

residue in which R^6 represents an alkyl residue having from 1 to 10 carbon atoms,

R^7 represents a hydrogen atom, a C_1-C_7 alkyl, hydroxymethyl, 1-hydroxyethyl, mercaptomethyl, 2-(methylthio)ethyl, 3-aminopropyl, 3-ureidopropyl, 3-guanidylpropyl, 4-aminobutyl, carboxymethyl, carbamoylmethyl, 2-carboxyethyl, 2-carbamoylethyl, benzyl, 4-hydroxybenzyl, 3-indolylmethyl or 4-imidazolylmethyl group,

R^8 represents a hydrogen atom or a methyl group, and

R^9 represents a hydrogen atom, an acetyl, benzoyl, trichloroacetyl, trifluoroacetyl, methoxycarbonyl, t-butyloxycarbonyl or benzyloxycarbonyl group, and

R^7 and R^8 may, when they are taken together, represent a $-CH_2-CH_2-CH_2-$ group.

2. Composition according to Claim 1, which comprises at least two compounds, a first compound being selected from the saponins purified from an extract of *Quillaja saponaria* and a second compound being selected from cationic lipids which are weak inhibitors of protein kinase C and have a structure which includes a lipophilic group derived from cholesterol, a bonding group selected from carboxyamides and carbamoyls, a spacer arm consisting of a branched or unbranched linear alkyl chain of 1 to 20 carbon atoms, and a cationic amine group selected from primary, secondary, tertiary and quaternary amines.

3. Composition according to Claim 1 or 2, in which the compound is a saponin which is the QS-21 fraction purified from a *Quillaja saponaria* extract.

4. Composition according to Claim 1 or 2, in which the compound is a cationic lipid made in the form of a liposome.

5. Composition according to Claim 1, 2 or 4, in which the compound is a cationic lipid which is 3-beta-[N-(N',N'-dimethylaminoethane)carbamoyl]cholesterol(DC-chol).

6. Composition according to Claim 1, in which the compound is a glycolipopeptide which is N-(2-L-leucin-amido-2-deoxy-β-D-glucopyranosyl)N-octadecyl-dodecanoylamide (Bay R1005).

7. Composition according to one of Claims 1 to 6, in which the immunogenic agent derived from *Helicobacter* is selected from a preparation of inactivated *Helicobacter* bacteria, a *Helicobacter* cell lysate, a peptide and a polypeptide from *Helicobacter* in purified form.

8. Composition according to Claim 7, in which the immunogenic agent derived from *Helicobacter* is the UreB or UreA subunit of *Helicobacter* urease.

9. Composition according to one of Claims 1 to 8, in which the immunogenic agent is derived from *Helicobacter pylori*.

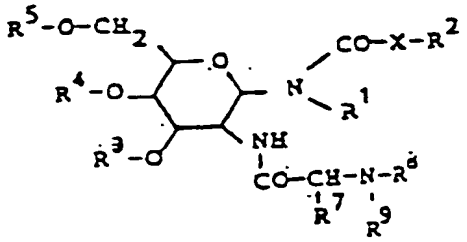
10. Use of an immunogenic agent derived from *Helicobacter* and of at least one compound selected from:

(i) saponins purified from an extract of *Quillaja saponaria*;

(ii) cationic lipids which are weak inhibitors of protein kinase C and have a structure which includes a lipophilic group derived from cholesterol, a bonding group selected from carboxyamides and carbamoyls, a spacer arm consisting of a branched or unbranched linear alkyl chain of 1 to 20 carbon atoms, and a

cationic amine group selected from primary, secondary, tertiary and quaternary amines; and

(iii) glycolipopeptides of formula (I):



(I)

in which

R^1 represents an alkyl residue saturated or unsaturated once or several times and comprising from 1 to 50 carbon atoms,

X represents $-CH_2-$, -O- or -NH-,

R^2 represents a hydrogen atom or an alkyl residue saturated or unsaturated once or several times and comprising from 1 to 50 carbon atoms,

R^3, R^4 and R^5 each represent, independently of each other, a hydrogen atom or an acyl-$CO-R^6$ residue in which R^6 represents an alkyl residue having from 1 to 10 carbon atoms,

R^7 represents a hydrogen atom, a C_1-C_7 alkyl, hydroxymethyl, 1-hydroxyethyl, mercaptomethyl, 2-(methylthio)ethyl, 3-aminopropyl, 3-ureidopropyl, 3-guanidylpropyl, 4-aminobutyl, carboxymethyl, carbamoylmethyl, 2-carboxyethyl, 2-carbamoylethyl, benzyl, 4-hydroxybenzyl, 3-indolylmethyl or 4-imidazolylmethyl group,

R^8 represents a hydrogen atom or a methyl group, and

R^9 represents a hydrogen atom, an acetyl, benzoyl, trichloroacetyl, trifluoroacetyl, methoxycarbonyl, t-butyloxycarbonyl or benzyloxycarbonyl group, and

R^7 and R^8 may, when they are taken together, represent a $-CH_2-CH_2-CH_2-$ group;

in the manufacture of a pharmaceutical composition capable of inducing a T helper 1 (Th1) type immune response against *Helicobacter*.

11. Use according to Claim 10, of an immunogenic agent derived from *Helicobacter* and of at least two compounds, a first compound being selected from the saponins purified from an extract of *Quillaja saponaria* and a second compound being selected from cationic lipids which are weak inhibitors of protein kinase C and have a structure which includes a lipophilic group derived from cholesterol, a bonding group selected from carboxyamides and carbamoyls, a spacer arm consisting of a branched or unbranched linear alkyl chain of 1 to 20 carbon atoms, and a cationic amine group selected from primary, secondary, tertiary and quaternary amines.

12. Use according to Claim 10 or 11, in which the compound is a saponin which is the QS-21 fraction purified from a *Quillaja saponaria* extract.

13. Use according to Claim 10 or 11, in which the compound is a cationic lipid made in the form of a liposome.

14. Use according to Claim 10, 11 or 13, in which the compound is 3-beta-[N-(N',N'-dimethylaminoethane)-carbamoyl]cholesterol (DC-chol).

15. Use according to Claim 10, in which the compound is a glycolipopeptide which is N-(2-L-leucin-

amido-2-deoxy -β-D-glucopyranosyl) N-octadecyl-dodecanoylamide (Bay R1005).

16. Use according to one of Claims 10 to 15, in which the Th1 type immune response is measured in mice and is characterized either (i) by a ratio of the ELISA IgG2a : IgG1 titres greater than or equal to 1 : 100 or (ii) by a ratio of the ELISA IgG2a : IgA titres greater than or equal to 1 : 100.

17. Use according to Claim 16, in which the Th1 type immune response is measured in mice and is characterized either (i) by a ratio of the ELISA IgG2a : IgG1 titres greater than or equal to 1 : 10 or (ii) by a ratio of the ELISA IgG2a : IgA titres greater than or equal to 1 : 10.

18. Use according to Claim 17, in which the Th1 type immune response is measured in mice and is characterized either (i) by a ratio of the ELISA IgG2a : IgG1 titres greater than or equal to 1 : 2 or (ii) by a ratio of the ELISA IgG2a : IgA titres greater than or equal to 1 : 2.

19. Use according to one of Claims 10 to 18, in which the immunogenic agent derived from *Helicobacter* is selected from a preparation of inactivated *Helicobacter* bacteria, a *Helicobacter* cell lysate, a peptide and a polypeptide from *Helicobacter* in purified form.

20. Use according to Claim 19, in which the immunogenic agent derived from *Helicobacter* is the UreB or UreA subunit of *Helicobacter* urease.

21. Use according to one of Claims 10 to 20, in which the immunogenic agent is derived from *Helicobacter pylori*.

22. Use according to one of Claims 10 to 21, in which the pharmaceutical composition is intended to be administered by the systemic route.

23. Use according to Claim 22, in which the pharmaceutical composition is intended to be administered by the strict systemic route.

24. Use according to Claim 22 or 23, in which the pharmaceutical composition is intended to be administered by the systemic route in the part of a mammal, in particular of a primate, situated under its diaphragm.

25. Use according to one of Claims 22 to 24, in which the pharmaceutical composition is intended to be administered by a systemic route in the dorsolumbar region of a mammal, in particular a primate.

26. Use according to one of Claims 22 to 25, in which the pharmaceutical composition is intended to be administered by a systemic route selected from the subcutaneous route, the intramuscular route and the intradermal route.

27. Use according to one of Claims 10 to 26, in which the pharmaceutical composition is intended to be administered twice or three times by the systemic route during the same treatment, to prevent or treat a *Helicobacter* infection.

28. Conjoint use of an immunogenic agent derived from *Helicobacter* and of a compound capable of promoting the induction of a T helper 1 (Th1) type immune response against *Helicobacter*, in the manufacture of a pharmaceutical composition intended to be administered by the systemic route to prevent or treat a *Helicobacter* infection.

Figure 1

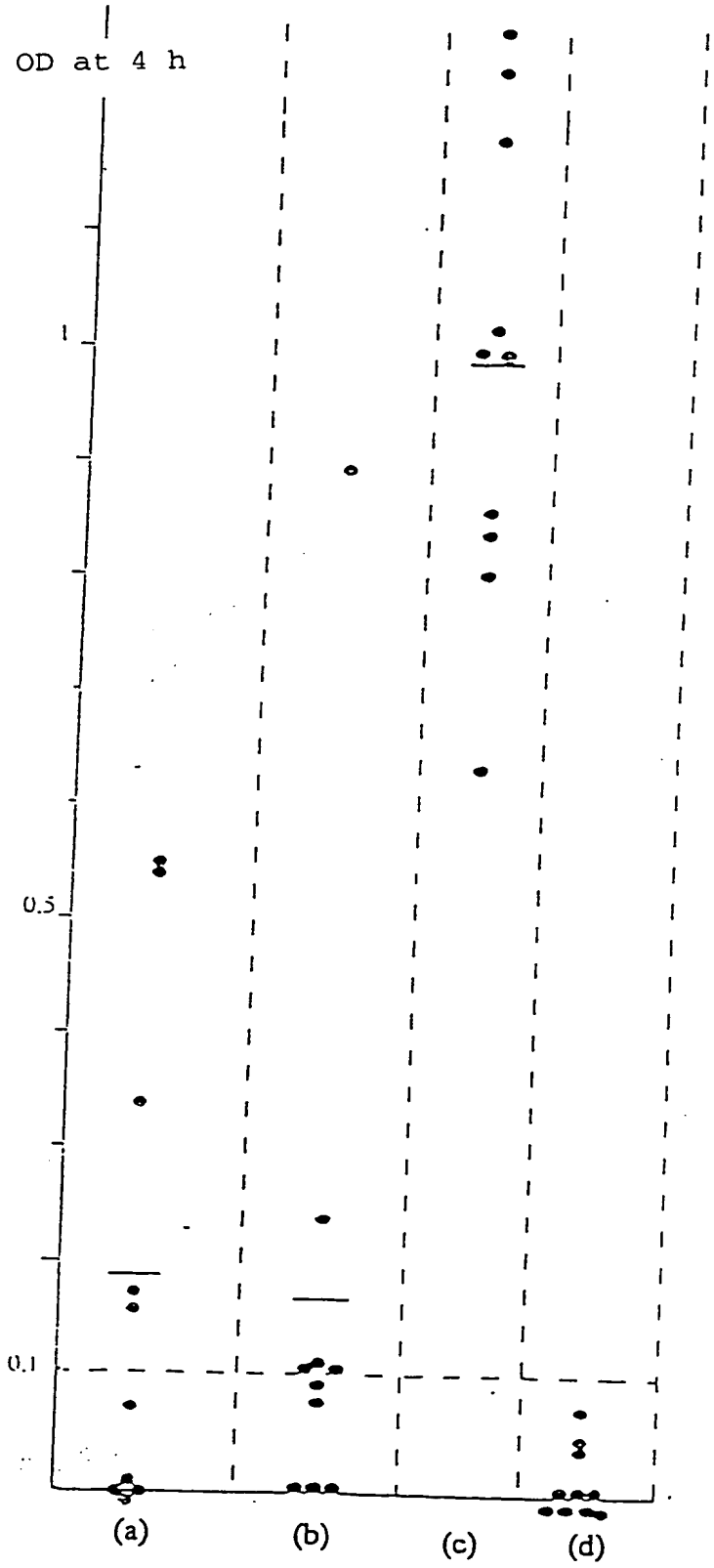


Figure 2

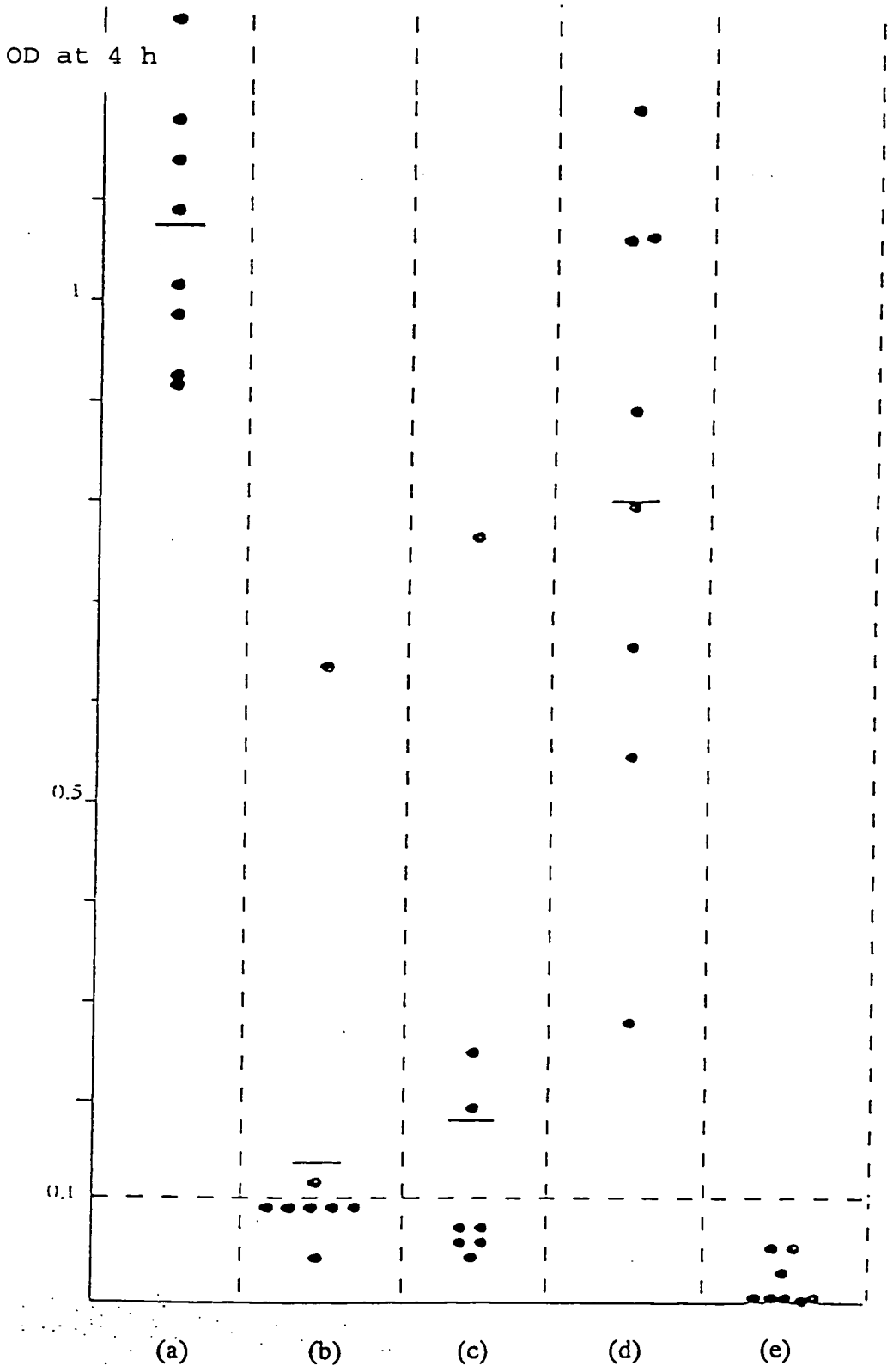


Figure 3

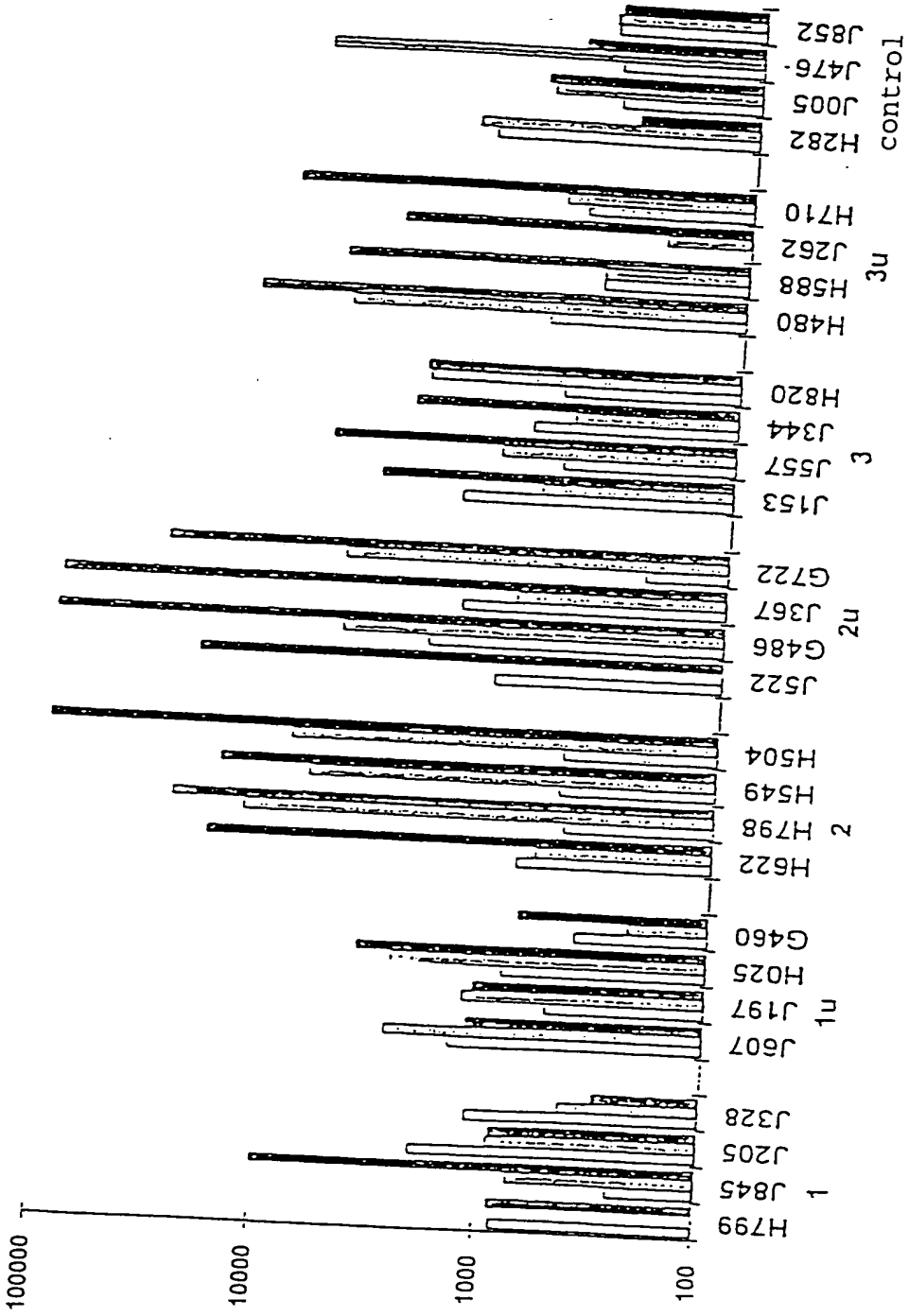

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Figure 4

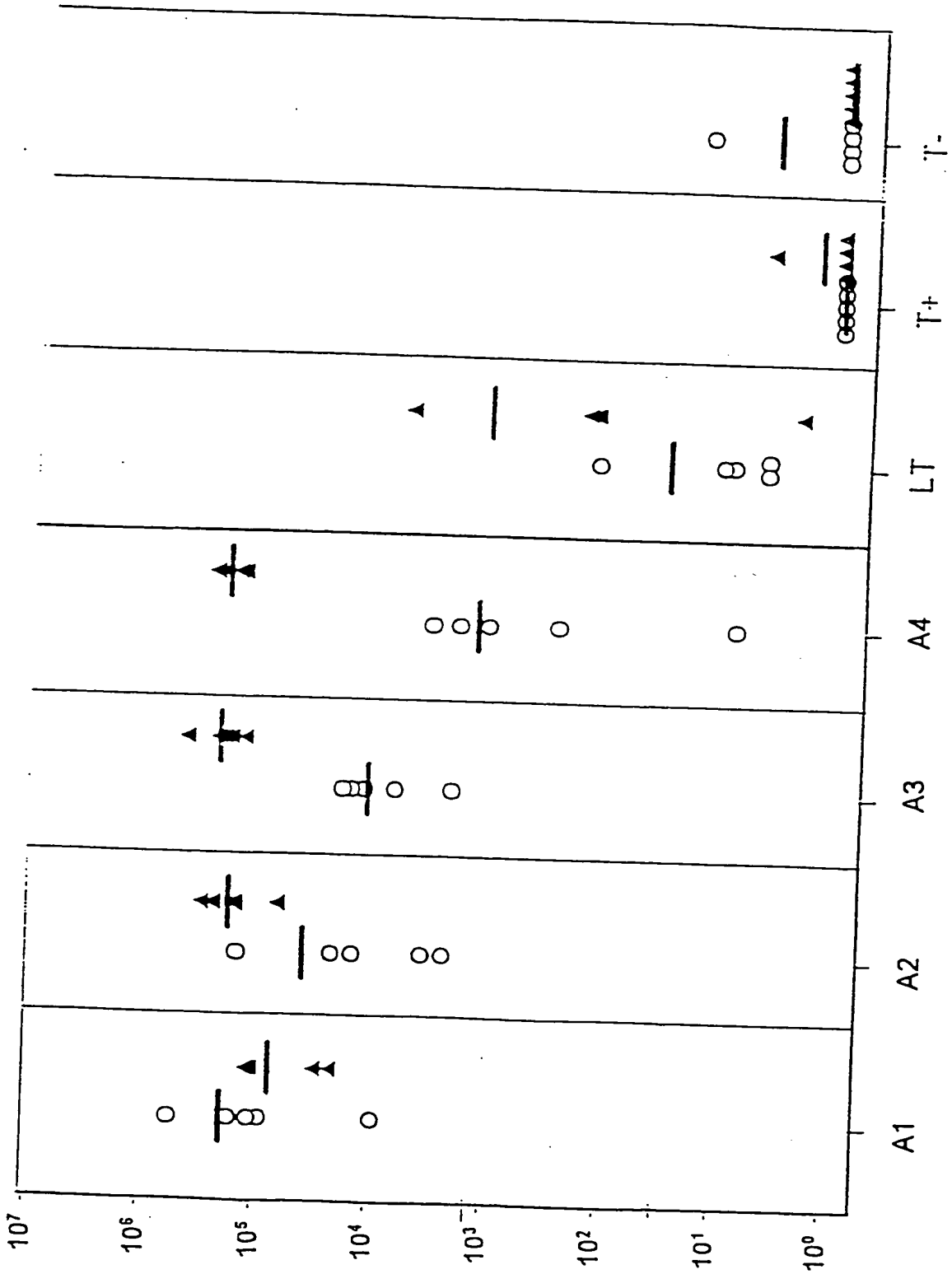

Figure 5

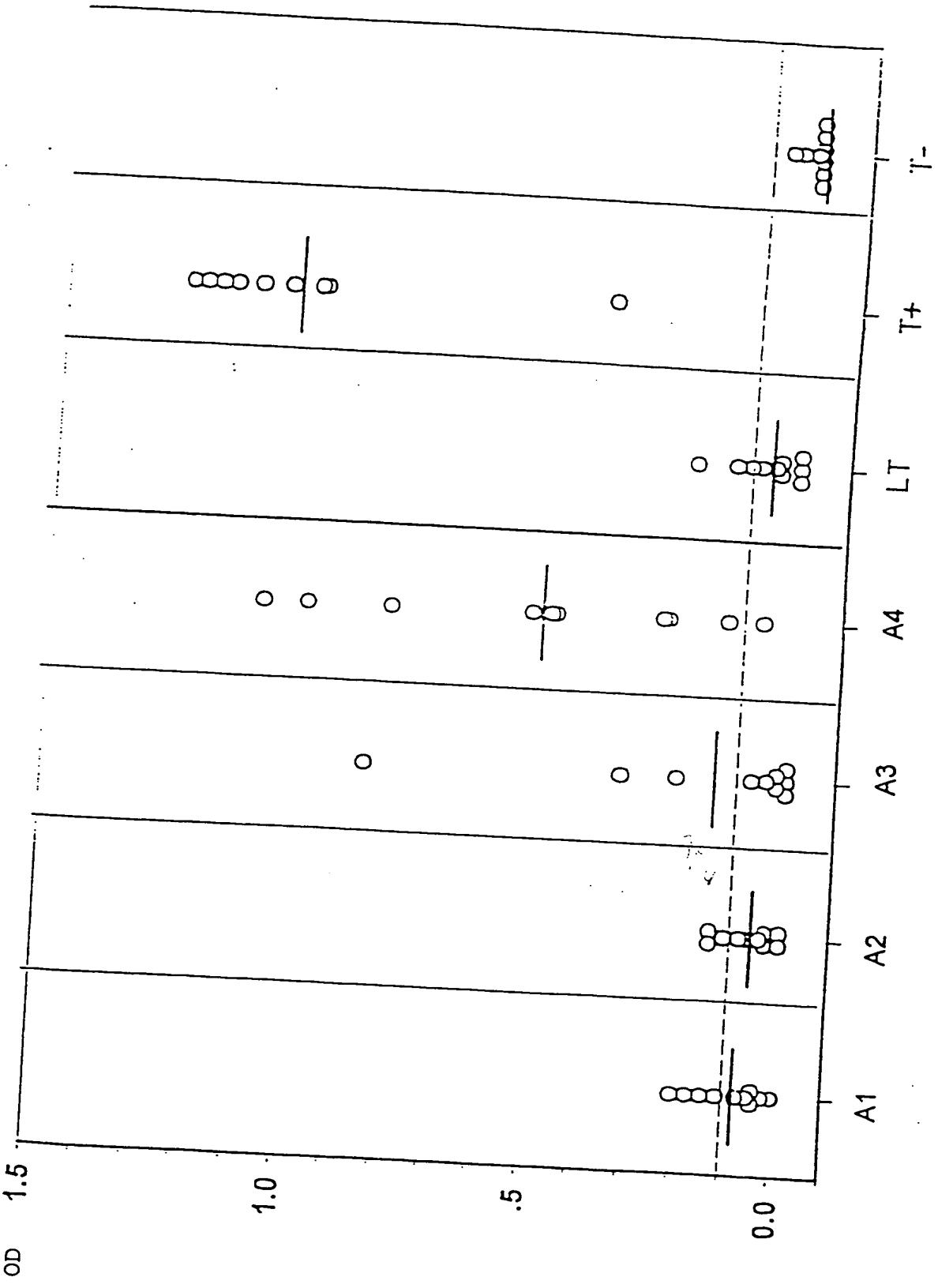

Figure 6

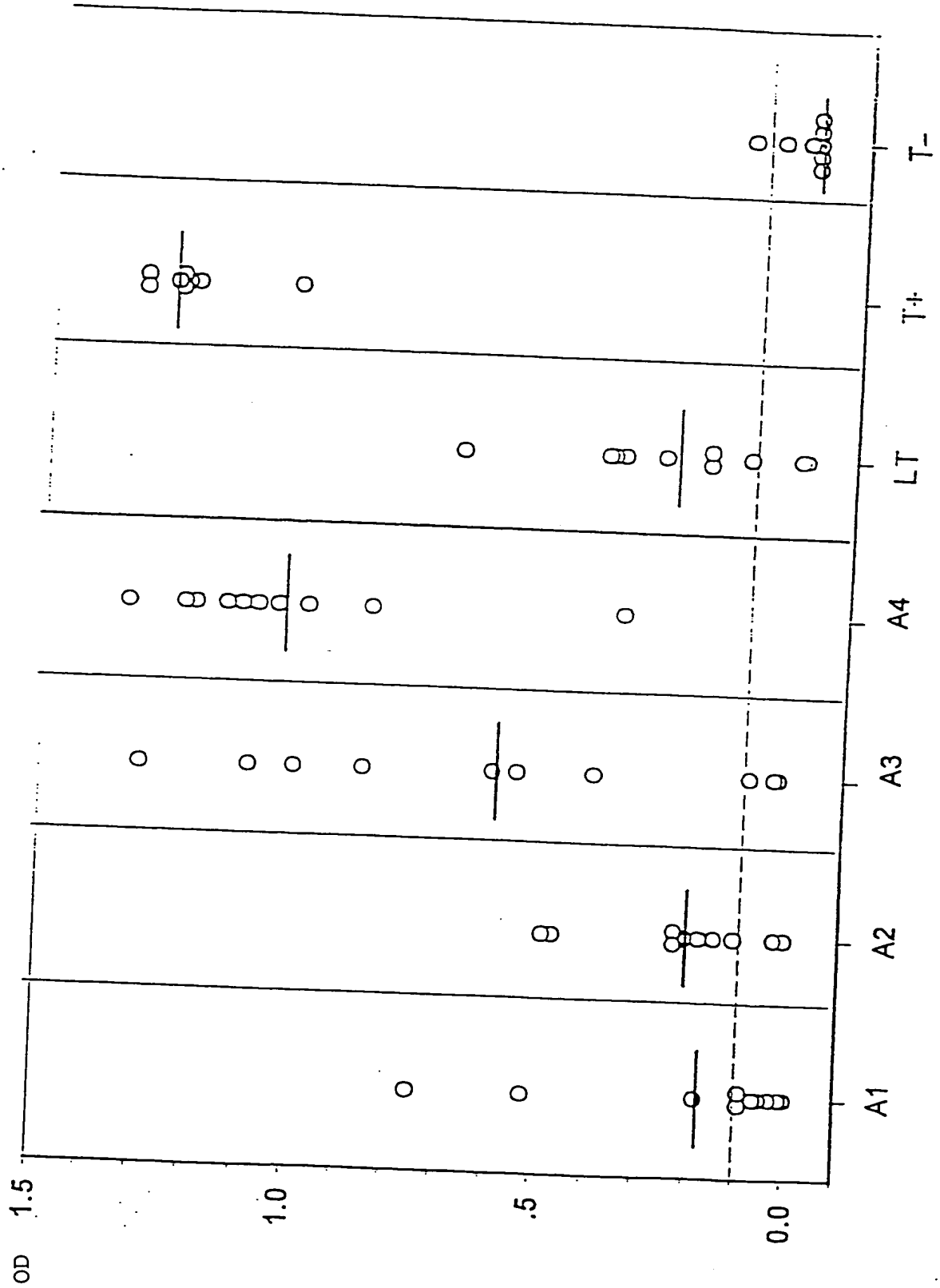

Figure 7